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CLONING, SEQUENCING, AND EXPRESSION OF HUMAN GONADOTROPIN RELEASING HORMONE (GnRH) RECEPTOR[1]

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Summary: Gonadotropin releasing hormone is a hypothalamic decapeptide that stimulates the release of gonadotropic hormones from the anterior pituitary gland. Therapeutically, the human pituitary GnRH receptor is the target of agonists used in the suppression of prostate cancer. Here we report the isolation of a cDNA representing this receptor. It encodes a protein with a transmembrane topology similar with that of other G protein-coupled, 7-transmembrane receptors. Binding studies of the cloned receptor demonstrate high affinity and pharmacological properties similar with the native human pituitary GnRH receptor. Northern blot and reverse transcriptase/PCR analysis revealed that its mRNA is expressed in pituitary, ovary, testis, breast, and prostate but not in liver and spleen. Availability of a human GnRH receptor cDNA should permit the design of improved analogs for therapeutic applications.

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide that is the key neural regulator of the reproductive process (1). It is synthesized by hypothalamic neurons, secreted in a pulsatile manner, and carried to the anterior lobe of the pituitary gland by way of the hypothalamo-hypophyseal portal circulation. There, GnRH stimulates secretion of the gonadotropic hormones (LH and FSH) which, in turn, regulate the gametogenic and hormonal functions of the gonads (1). The primary site of action of GnRH in the pituitary gland is the gonadotrope, the cell that expresses GnRH-receptors and secretes gonadotropic hormones (2). This receptor is an established member of the G-protein coupled, Ca^{2+}-dependent family of receptors (2). Receptors for GnRH also have been identified in other tissues of the rat such as the gonads (3), adrenal cortex (3), and brain (4) where their functions are not definitively established.

Most known characteristics of GnRH receptors have come from studies in the rat (2). In limited studies of the human GnRH receptor (5), investigators have shown it to differ from the rat receptor in molecular size (64 kD vs. 60 kD), in

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L03380.

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binding affinity for some GnRH antagonists, and sensitivity towards monovalent and divalent cations.

Paradoxically, exposure of the pituitary gland to supraphysiological concentrations of GnRH (6) or continuously to long acting GnRH agonists (7) causes down regulation and desensitization of GnRH receptors, leading to profound decreases in gonadotropin secretion. It is on this basis that analogs of GnRH are currently approved for treatment of precocious puberty, endometriosis, and prostate cancer in humans (7).

Recently, a cDNA clone for a GnRH receptor has been isolated from mouse anterior pituitary cell line (8). Using the mouse GnRH receptor cDNA as a probe, we have cloned, sequenced and expressed a GnRH receptor from the human pituitary gland.

Materials and Methods

cDNA cloning. The probe for screening the human pituitary cDNA library was mouse anterior pituitary GnRH receptor cDNA representing its open reading frame (8) prepared by reverse transcriptase/PCR (9, 10) as follows. Total RNA was prepared from mouse anterior pituitary gonadotrope cell line (αT3-1) by the guanidinium/CsCl method (11), and poly (A^+)-RNA was purified by oligo(dT)-cellulose chromatography. First strand cDNA was prepared using oligo(dT) primer and AMV reverse transcriptase following the protocol provided by the supplier (Promega). The primers used for PCR were sense 5'-ATGGCTAACAATGCATCTTG-3' and antisense 5'-CTACAAAGAGAAATACCCAT-3'(8).

A lambda gt10 cDNA library (12) prepared from human pituitary poly (A^+)-RNA primed with oligo(dT) (kindly donated by Dr. Olivier Civelli, Oregon Health Sciences University, Portland) was screened with ^{32}P-labeled mouse GnRH receptor cDNA. In brief, approximately 5 x 10^5 plaques from the library were plated and screened by plaque hybridization (13). Positive clones were purified to homogeneity. Inserts from the positive clones were excised with EcoR1 and subcloned into the pcDNA-I vector. Nucleotide sequencing of three clones was performed completely on both strands using the Sequenase 2.0 kit (US Biochemical) and synthetic primers. The sequence analysis presented was performed using the Wisconsin GCG program on a VAX computer.

Transfection of COS-7 cells with human GnRH receptor cDNA for binding assays. COS-7 cells grown on 60 mm Petri dishes were transfected with 5.0 ug of CsCl-purified plasmid DNA (pcDNA-I) bearing the human GnRH receptor cDNA using 60 μl of lipofectin as described previously (9). After 60 to 72 hr, the cells were washed with PBS, scraped-off the dishes, and homogenized in buffer (10 mM Tris-HCl, pH 7.4) to prepare the cell membranes for GnRH receptor radioassay as described by Wormald *et al*. (5) for human pituitary. The radioligand was ^{125}I [D-Ala^6] des-Gly^{10}-GnRH. Nonspecific binding was determined in the presence of 10^{-6}M [D-Ala^6]des-Gly^{10}-GnRH (GnRH-A, a GnRH agonist).

Calcium measurements. COS-7 cells were transfected with GnRH receptor cDNA as described above, collected after 65 hr, and attached to glass coverslips coated with poly-L-lysine. Calcium measurements in these cells were performed using Fura-2 as previously described (9).

Tissue expression of human GnRH receptor mRNA. Normal human tissues were obtained from The Tissue Procurement Facility, Comprehensive Cancer Center, University of Alabama at Birmingham. Such tissues are collected at the time of autopsy (3-12 hr after death) or biopsy and are immediately frozen and stored in liquid nitrogen. Total RNA from human pituitary, ovary, testis, breast,

prostate, liver, and spleen was prepared as described earlier. Twenty μg of RNA from each tissue was separated on a 1.0% agarose gel. Northern blot analysis was performed under high stringency conditions using random hexamer primed ^{32}P-labeled full length human GnRH receptor (1.5 kb) as previously described (14). For reverse transcriptase/PCR analysis of GnRH receptor mRNA, we prepared first strand cDNA using 2 μg of the total RNA in a 20 μl reaction volume as described earlier. A five μl aliquot of this solution was then used in a polymerase chain reaction (PCR) using the GeneAmp kit. The primers used were sense 5'-GCTTGAAGCTCTGTCCTGGGA-3' (overline, Fig. 1) and antisense 5'-CCTAGGACATAGTAGGG-3'(underline, Fig. 1). The reaction conditions were 1.5 min at 95°C, 1.5 min at 54°C, and 2 min at 72°C for 30 cycles in a Perkin-Elmer Cetus DNA thermal cycler. Twenty μl of the 100 μl reaction mixture was then electrophoresed through a 1.2% agarose gel and stained with ethidium bromide.

Results and Discussion

Ten cDNA clones that hybridized with mouse GnRH receptor cDNA were isolated from the human pituitary cDNA library. The EcoRI inserts from 4 of these clones were subcloned into the pcDNA-I vector for sequencing and eukaryotic expression. Three of the 4 clones contained complete open reading frames and hence were completely sequenced on both strands; their sequences were identical. The nucleotide sequence and corresponding predicted amino acid sequence of the GnRH receptor (hGnRHR) cDNA are shown in Fig. 1. The cDNA is composed of approximately 1550 nucleotides and contains an apparent poly(A^+)-tail at the 3'-end. Due to the absence of a polyadenylation signal, we assume that this poly(A^+)-region is not a poly(A^+)-tail but is sufficiently long that oligo(dT) priming occurred in this region. The open reading frame encodes a 328 amino acid protein (relative Mr=37,730). The nucleotide sequence exhibits 75% identity with the mouse GnRH receptor cDNA overall and 85% identity with its open reading frame (8); there is 89% identity at the amino acid level between the two receptors (8). Hydropathic analysis of the predicted protein identified seven stretches of highly hydrophobic amino acids (Fig. 2). The protein contains several potential sites for post-translational modification, which include two N-linked glycosylation sites; four cysteine residues, and; five serine and threonine residues in the cytoplasmic domain that are candidates for regulatory phosphorylation (Fig. 2). The receptor lacks a C-terminal cytoplasmic domain which also was reported for the mouse GnRH-receptor (8); this is unprecedented among 7-transmembrane, G-protein coupled receptors (15), and may relate to this receptor's propensity to desensitize.

GnRH receptor radioassays performed on transfected COS-7 cells using ^{125}I[D-Ala^6]des-Gly^{10}-GnRH, demonstrate that the cDNA encodes a GnRH receptor with high affinity (16, 17). The IC_{50} values for various ligands were as follows (Fig. 3): [D-Ala^6]des-Gly^{10}-GnRH (GnRH-A), 0.6×10^{-9}M; GnRH, 5×10^{-9}M; [Ac-D-pCl-Phe^{1,2},D-Trp^3,D-Arg^{6}D-Ala^{10}]GnRH (GnRH-Ant 1), 1×10^{-9}M; [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH (GnRH-Ant 2), 4×10^{-9}M, and; [D-Phe^{2,6},Pro^3]GnRH (GnRH-Ant 3), 50×10^{-9}M. Wormald et al. (5) performed GnRH receptor radioassays on human pituitary cell membranes

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      -55 GTTGTGCTGTCAGTGCACCAGAGACACAAGGCTTGAAGCTCTGTCCTGGGAAAAT   -1

          ATGGCAAACAGTGCCTCTCCTGAACAGAATCAAAATCACTGTTCAGCCATCAACAACAGC
        1 M  A  N  S  A  S  P  E  Q  N  Q  N  H  C  S  A  I  N  N  S    60
          ATCCCACTGATGCAGGGCAACCTCCCCACTCTGACCTTGTCTGGAAAGATCCGAGTGACG
       21 I  P  L  M  Q  G  N  L  P  T  L  T  L  S  G  K  I  R  V  T   120
          GTTACTTTCTTCCTTTTTCTGCTCTCTGCGACCTTTAATGCTTCTTTCTTGTTGAAACTT
       41 V  T  F  F  L  F  L  L  S  A  T  F  N  A  S  F  L  L  K  L   180
          CAGAAGTGGACACAGAAGAAAGAGAAAGGGAAAAAGCTCTCAAGAATGAAGCTGCTCTTA
       61 Q  K  W  T  Q  K  K  E  K  G  K  K  L  S  R  M  K  L  L  L   240
          AAACATCTGACCTTAGCCAACCTGTTGGAGACTCTGATTGTCATGCCACTGGATGGGATG
       81 K  H  L  T  L  A  N  L  L  E  T  L  I  V  M  P  L  D  G  M   300
          TGGAACATTACAGTCCAATGGTATGCTGGAGAGTTACTCTGCAAAGTTCTCAGTTATCTA
      101 W  N  I  T  V  Q  W  Y  A  G  E  L  L  C  K  V  L  S  Y  L   360
          AAGCTTTTCTCCATGTATGCCCCAGCCTTCATGATGGTGGTGATCAGCCTGGACCGCTCC
      121 K  L  F  S  M  Y  A  P  A  F  M  M  V  V  I  S  L  D  R  S   420
          CTGGCTATCACGAGGCCCCTAGCTTTGAAAAGCAACAGCAAAGTCGGACAGTCCATGGTT
      141 L  A  I  T  R  P  L  A  L  K  S  N  S  K  V  G  Q  S  M  V   480
          GGCCTGGCCTGGATCCTCAGTAGTGTCTTTGCAGGACCACAGTTATACATCTTCAGGATG
      161 G  L  A  W  I  L  S  S  V  F  A  G  P  Q  L  Y  I  F  R  M   540
          ATTCATCTAGCAGACAGCTCTGGACAGACAAAAGTTTTCTCTCAATGTGTAACACACTGC
      181 I  H  L  A  D  S  S  G  Q  T  K  V  F  S  Q  C  V  T  H  C   600
          AGTTTTTCACAATGGTGGCATCAAGCATTTTATAACTTTTTCACCTTCAGCTGCCTCTTC
      201 S  F  S  Q  W  W  H  Q  A  F  Y  N  F  F  T  F  S  C  L  F   660
          ATCATCCCTCTTTTCATCATGCTGATCTGCAATGCAAAAATCATCTTCACCCTGACACGG
      221 I  I  P  L  F  I  M  L  I  C  N  A  K  I  I  F  T  L  T  R   720
          GTCCTTCATCAGGACCCCCACGAACTACAACTGAATCAGTCCAAGAACAATATACCAAGA
      241 V  L  H  Q  D  P  H  E  L  Q  L  N  Q  S  K  N  N  I  P  R   780
          GCACGGCTGAAGACTCTAAAAATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGG
      261 A  R  L  K  T  L  K  M  T  V  A  F  A  T  S  F  T  V  C  W   840
          ACTCCCTACTATGTCCTAGGAATTTGGTATTGGTTTGATCCTGAAATGTTAAACAGGTTG
      281 T  P  Y  Y  V  L  G  I  W  Y  W  F  D  P  E  M  L  N  R  L   900
          TCAGACCCAGTAAATCACTTCTTCTTTCTCTTTGCCTTTTTAAACCCATGCTTTGATCCA
      301 S  D  P  V  N  H  F  F  F  L  F  A  F  L  N  P  C  F  D  P   960
          CTTATCTATGGATATTTTTCTCTGTGATTGATAGACTACACAAGAAGTCATATGAAGAAG
      321 L  I  Y  G  Y  F  S  L  #                                    1020

          GGTAAGGTAATGAATCTCTCCATCTGGGAATGATTAACACAAATGTTGGAGCATGTTTAC 1080
          ATACAAACAAAGTAGGATTTACACTTAAGTTATCATTCTTTTAGAAACTCAGTCTTCAGA 1140
          GCCTCAATTATTAAGGAAAAGCTCTTCAGGAAAAATACTAAAATATTTCTCTTCCTCATA 1200
          AGCTTCTAAATTAATCTCTGCCTTTTCTGACCTCATATAACACATTATGTAGGTTTCTTA 1260
          TCACTTTCTCTTTGCATAATAATGTACTAATATTTAAAATACCTTCAGCCTAAGGCACAA 1320
          GGATGCCAAAAAAACAAAGGTGAGAAACCACAACACAGGTCTAAACTCAGCATGCTTTGG 1380
          TGAGTTTTTCTCCAAAAGGGGCATATTAGCAATTAGAGTTGTATGCTATATAATACATAG 1440
          AGCACAGAGCCCTTTGCCCATAATATCAACTTTCCCTCCTATAGTTAAAAAAAAAAAAAA 1500
          AA                                                           1502
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FIG. 1. Nucleotide sequence of the human GnRH receptor cDNA and deduced amino acid sequence of the protein. The nucleotide sequence is listed above and numbered on the right while the amino acid sequence is listed below and numbered on the left. The overline (sense) and underline (antisense) designate the sequences of synthetic oligonucleotides used for the polymerase chain reactions shown in Fig. 5.

and reported values of 4.8 nM for GnRH and 4.2 nM for Ant 2 which are highly similar with our values of 5 and 4 nM, respectively. Finally, thyrotropin releasing hormone (TRH) and angiotensin II (AII) were virtually inactive (Fig. 3). Thus, the cDNA we have isolated encodes a protein with the pharmacological characteristics expected of the human GnRH receptor.

The GnRH receptor in cells from the anterior pituitary of rats has been shown to functionally couple to increases in intracellular Ca^{2+} (2). GnRH-dependent increases in intracellular Ca^{2+} also have been demonstrated with the cloned mouse GnRH receptor, when RNA transcribed from the cDNA was injected into Xenopus oocytes (8). To determine whether Ca^{2+} serves as the second messenger for the cloned human GnRH receptor, we used COS-7 cells transfected with the GnRH receptor cDNA for measurement of $(Ca^{2+})_i$ levels using video-based fluorescence

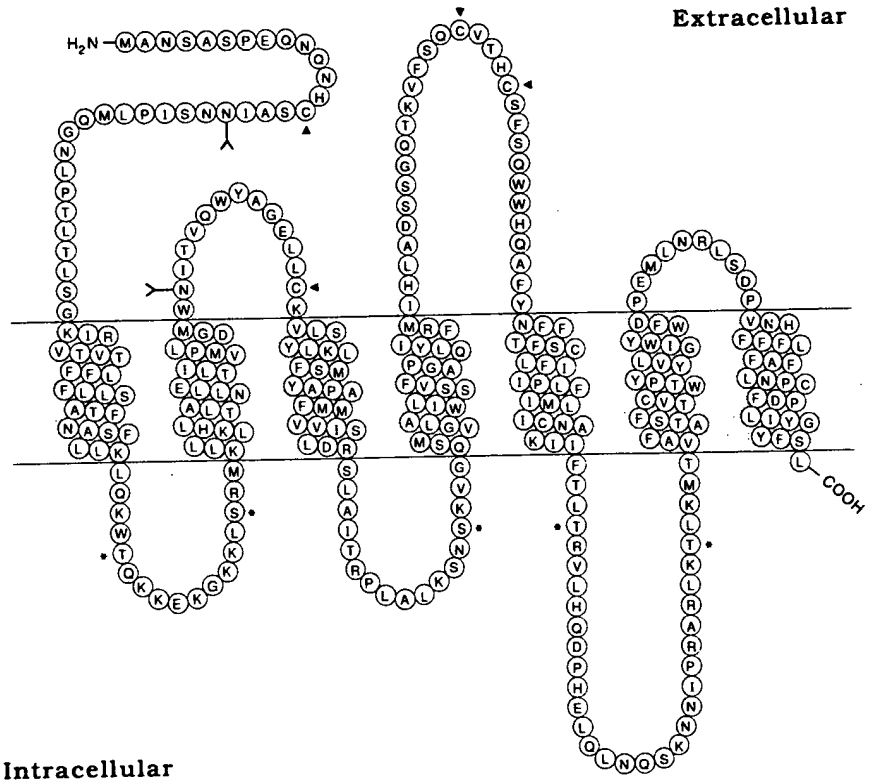

FIG. 2. Proposed seven transmembrane topography of the human GnRH receptor. The shaded area represents the cell membrane. Also indicated are the canonical sites of N-linked glycosylation (Y), potential sites for phosphorylation by protein kinase C (*), and cysteine residues in the extracellular loops (▼).

ratio imaging of the calcium indicator dye, Fura-2, as described previously (9). About 12% of the transfected COS-7 cells responded to 10^{-7}M GnRH-A and they exhibited a mean 2.0-fold increase in the Fura-2 fluorescence ratio (Fig. 4).

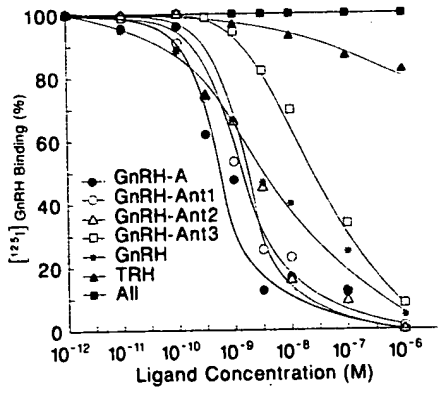

FIG. 3. Unlabelled ligand displacement curves in an ^{125}I-[D-Ala^6]-des-Gly^{10}-GnRH receptor radioassay performed on membranes from COS-7 cells transfected with the human GnRH receptor cDNA. The results shown are the mean values derived from 2-4 assays derived from independent transfections. See text for definition of the abbreviations of the ligands.

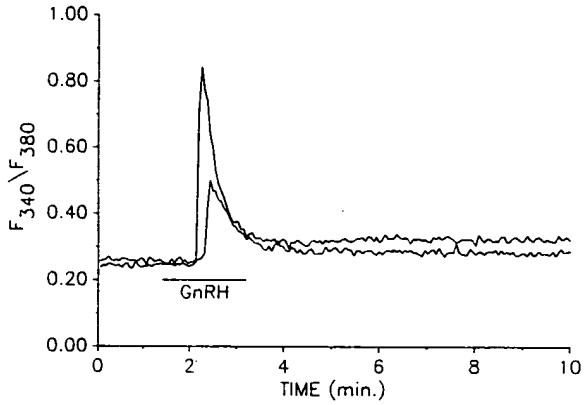

FIG. 4. GnRH-A-stimulated increase in intracellular Ca^{2+} levels in two COS-7 cells transfected with the human GnRH receptor cDNA. In 2 independent transfection assays, 20 of 165 COS-7 cells (12.1%) responded to GnRH-A (10^{-7}M); none of 60 cells responded to GnRH-A when transfected with pcDNA-I plasmids carrying the human GnRH receptor cDNA in the antisense orientation.

None of the COS-7 cells transfected with a plasmid bearing the GnRH receptor cDNA cloned in the opposite direction responded to GnRH-A. Thus, the human GnRH receptor (hGnRHR) also appears to be functionally coupled to increases in intracellular Ca^{2+}.

Tissue distribution and size of the human GnRH receptor were determined in high stringency Northern blot hybridizations using as a probe a random hexamer primed, full length hGnRHR cDNA (1.5 kb EcoRI insert from the λgt10 clone). This analysis revealed a 5.0 kb mRNA in human pituitary but not in other tissues (ovary, testis, breast, prostate) (data not shown). As an additional test of whether GnRH mRNA is expressed in tissues other than pituitary, we used the more sensitive reverse transcriptase/PCR technique. As shown in Fig. 5, PCR products corresponding to the expected size of the GnRH receptor (800 bp) are clearly visible in human pituitary, ovary, testis, breast, and prostate and in the MCF-7 breast cancer cell line (Fig. 5) but not in liver and spleen (data not shown). Although our RT-PCR assay is only semi-quantitative, expression of GnRH receptor was highest in pituitary compared to other tissues (Fig. 5).

In summary, a human GnRH receptor cDNA has been cloned, sequenced, and expressed. Pharmacological and functional characterization of the receptor

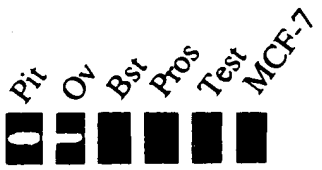

FIG. 5. Human GnRH receptor mRNA expression in various tissues and a breast tumor cell line. Ethidium bromide stained DNA is shown which was generated from tissue or cell mRNA using reverse transcriptase/polymerase chain reactions. Abbreviations: Pit, anterior pituitary gland; Ov, ovary; Bst, breast; Pros, prostate gland; Test, testis; MCF-7, breast tumor cell line.

support the conclusion that the cloned receptor is highly similar if not identical with the native receptor in the human pituitary. The availability of this receptor cDNA will facilitate the study of its role in reproduction and cancer, and will contribute to the development of improved GnRH receptor-specific agonists and antagonists for human therapy.

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